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DIRECT AND INDIRECT CAUSAL EFFECTS OF HETEROZYGOSITY ON FITNESS-RELATED TRAITS IN ALPINE IBEX

Alice Brambilla[1], Iris Biebach[2], Bruno Bassano[3], Giuseppe Bogliani[1], Achaz von Hardenberg[3]

[1] DSTA - Department of Earth and Environmental Science, University of Pavia, Via A. Ferrata 9, 27100 Pavia (PV), Italy.

[2] Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland.

[3] Alpine Wildlife Research Centre, Gran Paradiso National Park, Degioz 11, 11010 Valsavarenche (AO), Italy.

Corresponding author: Alice Brambilla
DSTA- Department of Earth and Environmental Science, University of Pavia, Via A. Ferrata 9, 27100 Pavia (PV), Italy.
Tel. +39.3289230658
E-mail address: alicebrambilla1@gmail.com

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Abstract

Heterozygosity-fitness correlations (HFCs) are a useful tool to investigate the effects of inbreeding in wild populations, but are not informative in distinguishing between direct and indirect effects of heterozygosity on fitness-related traits. We tested HFCs in male Alpine ibex (*Capra ibex*) in a free-ranging population, which suffered a severe bottleneck at the end of 18th century and used confirmatory path analysis to disentangle the causal relationships between heterozygosity and fitness-related traits. We tested HFCs in 149 male individuals born between 1985 and 2009. We found that standardized multilocus heterozygosity (MLH), calculated from 37 microsatellite loci, was related to body mass and horn growth, which are known to be important fitness-related traits, and to faecal egg counts of nematode eggs (FEC), a proxy of parasite resistance. Then, using confirmatory path analysis, we were able to show that the effect of MLH on horn growth was not direct but mediated by body mass and FEC. Heterozygosity-fitness correlations do not necessarily imply direct genetic effects on fitness-related traits, which instead can be mediated by other traits in complex and unexpected ways.

Keywords: HFCs, inbreeding depression, multi-locus heterozygosity, bottleneck, confirmatory path analysis.

Introduction

Inbreeding is defined as the mating between closely related individuals and the consequent increase in homozygosity caused by such mating [1]. In small and isolated populations [2,3] and in bottlenecked populations with small effective population size [4], inbreeding can not be avoided. The consequence of mating between relatives may be the increase in expression of deleterious recessive alleles that lead to inbreeding depression: the decrease in fitness of inbred individuals compared to outbred individuals [5]. The detrimental effects of inbreeding were first summarized by Darwin [6]. Since then, evidence of the negative impact of inbreeding on fitness and viability at both individual and population levels has accumulated [1,7-10]. The expression of recessive deleterious mutations has been indicated as the main cause of inbreeding depression [11,12,13]. The negative effects of inbreeding might be purged through selection against deleterious alleles [14]. A review conducted on mammals, however, revealed contrasting results, with purging not substantially reducing inbreeding depression [15]. The efficiency of purging depends on many genetic and environmental factors [1], and the time necessary to markedly weaken inbreeding depression could be highly variable and very long [12,16].

Inbreeding depression is a relative measure of the difference in fitness between inbred and outbred individuals. If the variance of the inbreeding coefficient (f) in the population is low, then all individuals may suffer from the effects of inbreeding but they would all have similar fitness, and hence, inbreeding depression would not be detectable [12]. Thus, it is generally difficult to detect the effects of inbreeding depression in small and isolated or in bottlenecked populations with similar levels of inbreeding in all the individuals [13].

When complete pedigree data are available, inbreeding depression is usually measured by regressing fitness on inbreeding coefficient f [17]. Pedigree data, however, are difficult to obtain in wild populations. Moreover, pedigrees are typically relatively short and thus may miss inbreeding

69 that accumulated prior to the beginning of the pedigree, a fact of particular importance in
70 bottlenecked populations [10]. In the absence of pedigree data, molecular measures such as
71 heterozygosity have been used as a proxy of inbreeding [18]. A molecular-based method widely
72 used to test for inbreeding depression is heterozygosity-fitness correlations (HFCs): the correlation
73 between multilocus heterozygosity and fitness-related traits [12,13]. Whether HFCs are a signal of
74 inbreeding depression has been a matter of debate, but in the last years, a general consensus on this
75 hypothesis has been reached [12,13,18]. There are three main hypotheses invoked to explain HFCs
76 [19]: i) the direct effect hypothesis: the markers used to estimate heterozygosity are themselves
77 expressed and have a direct effect on fitness; ii) the local effect hypothesis: the markers used to
78 estimate heterozygosity are linked with loci directly affecting fitness; iii) the general effect
79 hypothesis: heterozygosity at microsatellite markers reflect genome-wide heterozygosity. Following
80 the general effect hypothesis, HFCs can be considered as a proxy of inbreeding depression in wild
81 populations [12]. HFCs are usually weak [12,20] and their detection depends on sample size and on
82 the number of available markers: an absence of HFCs is, thus, not necessarily an indication of
83 absence of inbreeding depression.
84
85 While HFCs are a useful tool to investigate the effects of inbreeding in wild populations, they are
86 not informative in distinguishing between direct and indirect effects of heterozygosity on fitness-
87 related traits. This is due to the very nature of the multiple regression models commonly used to test
88 HFCs, which do not allow causal inference. Controlled or randomized experiments are the
89 straightforward method to test for causal relationships among variables [21]. Experimental
90 manipulations, however, are very difficult if not impossible to conduct with large, free living
91 vertebrates that are part of long term observational studies. Confirmatory path analysis, a special
92 case of the more general structural equation models, is a causal inference method which can be used
93 with observational data. This inference tool, recently introduced to biologist by Shipley [22] makes

94 it possible to formulate complex causal models represented as directed acyclic graphs (DAG)
95 depicting the hypothesized causal relationships among all involved variables [23]. Each
96 hypothesized DAG can be defined by the implied conditional independencies due to the assumed
97 causal links among the variables, which can then be statistically tested by translating them into
98 structural equations [21,24]. While confirmatory path analysis and structural equation models have
99 widely been applied in general ecology [25,26], eco-physiology [27,28] and in evolutionary biology
100 [29], we are not aware of any study using them in the field of molecular ecology.
101 The Alpine ibex (*Capra ibex*) is a mountain ungulate currently distributed over the whole European
102 Alps. The species owes its current distribution to reintroduction programs, which used the Gran
103 Paradiso population in the north-western Italian Alps as the primary source [30]. The species almost
104 went extinct in the 18th century with less than 100 individuals left in the Gran Paradiso area [31,32].
105 Even if the current conservation status of Alpine ibex is considered as "Least concern" in the IUCN
106 Red List of Threatened Species [33], its recent demographic history [34] and its generally low
107 genetic variability [35,36] suggest this species is worth special attention. Inbreeding depression is
108 expected to particularly affect the isolated and recently reintroduced populations founded by few
109 individuals [37], but it probably also affects the original bottlenecked population in Gran Paradiso
110 [38]; in fact, the main genetic bottleneck event suffered by the population at the beginning of the
111 19th century [36] is quite recent in genetic times considering the long generation times that
112 characterize this species (about 7-8 years), and the reduced effective population size is expected to
113 be enough for HFCs to arise [12,13] also in the original population. Better knowledge of the effects
114 of inbreeding in Alpine ibex is, thus, important to understand how long the effects of a bottleneck
115 can last in a long lived mammal, and for conservation purposes.
116 A previous study carried out on the same population [38] only found a weak age-dependent effect
117 of heterozygosity on horn length. The sample size, however, was probably not large enough to
118 detect the presence of inbreeding depression. Moreover, this study [38] as well as other studies that

investigated HFCs in vertebrates, limited their analysis to the finding of correlations between heterozygosity and one or more fitness related traits, considering each trait as independently correlated to heterozygosity [10,39-42]. To the best of our knowledge, no study has tried to disentangle the possible causal relationships between heterozygosity and multiple fitness-related traits. In this study, we tested HFCs in individually tagged male Alpine ibex in the autochthonous population of Gran Paradiso National Park (GPNP, Italy) using a dataset collected over 14 years. We tested the effects of standardized multilocus heterozygosity (MLH) at 37 neutral microsatellite loci on various fitness-related traits: body mass, annual horn growth (named hereafter only 'horn growth') and faecal egg counts (FEC) of nematode parasites. We used confirmatory path analysis [22,24] to test alternative models of possible direct and indirect cause-effect relationships between MLH and fitness-related traits.

Methods

Study area and Population

This study was conducted in the Levionaz study area in Gran Paradiso National Park (GPNP; North-western Italian Alps; 45° 25' N, 07° 34' W) within the framework of a long-term project started in 1999 on the ecology and life history of the species [38,43-45]. Most male Alpine ibex in the Levionaz study area have been captured once and individually marked with colored ear tags. The percentage of marked males in Levionaz is around 80% (data of 2012, pers. obs.); cohort of marked animals ranged between 1985 and 2009 providing 25 years of molecular data. For more details on the marking protocol, see Brambilla et al. [46]. The capture and marking protocol used in this study has been authorized by the Italian Ministry of Environment after review by the Italian National Institute for Environmental Protection and Research (ISPRA).

Genetic analysis

Samples for genetic analysis were collected as tissue or blood samples during captures. We performed microsatellite analysis on samples from n= 149 different male Alpine ibex. After collection, tissue samples were stored in 95% ethanol solution, and blood samples were stored in EDTA vacutainer tubes at a temperature of -35°C or applied on absorbent paper cards for blood DNA (Whatman FTA ® cards) and stored at room temperature until analysis. DNA extraction was performed using QIAmp DNA mini and Biosprint 96 systems (Qiagen) applying the appropriate protocol for each type of sample. We genotyped all samples at 51 polymorphic microsatellite loci as originally described in Biebach and Keller [30]. Fragment analysis was performed on an ABI 3730 automated sequencer and electropherograms were analysed and manually checked using GeneMapper 4.0 (Applied Biosystems). PCR and genotyping was repeated up to 3 times, and a consensus genotype was built. PCR and genotyping were done twice for all blood samples that were stored on absorbent paper cards (n= 55) and once for all tissue and blood samples in EDTA (n= 94). The genotyping process was repeated if genotype quality was low (low intensity compared to other samples of the same marker) or if the two repetitions did not match. The genotype of a sample with three repetitions was considered heterozygote if at least 2 of the 3 repetitions were heterozygote and was considered homozygote if all repetitions were homozygote. Locus specific dropout and false allele rates were calculated using the software Gimlet [47], and then were compared with error rates found in another study of the same species [30]. Allelic drop-out and false allele rates are provided as supplementary material (Table S1).

Fitness-related traits data collection

In Levionaz, male Alpine ibex were repeatedly weighed during summer (late May-September) with an electronic platform scale baited with salt [43]. To allow comparison between individuals measured at different times of the year, body mass on the 1st of August in each year was estimated [48]; n= 391 records of August weight were available to test the relationship between

heterozygosity and body mass.

Ibex horns grow continuously throughout life. Annual growth is easily visible thanks to the rings that form because of the lack of horn growth during winter. The annual horn growth of male ibex was measured along a central line on the external side of both horns using a calliper to the nearest mm during captures or when the animals were found dead. In the years after captures, the annual horn growth of marked individuals (annuli) was measured from remote pictures following Bergeron [45] or Brambilla and Canedoli [49]. For each pair of annuli, horn growth was estimated as the mean value of the left and right annulus. We estimated horn growth for a total of n= 873 annuli. Plots showing the pattern of body mass and annual horn growth in function of age are provided in supplementary materials (Figure S1 and S2).

Faecal samples from marked individuals were collected once a month from May to September to determine individual faecal egg counts (FEC). A total of n= 510 measures of FEC were available. FECs have been used as a proxy of resistance to abomasal nematode infection (Abomasal trichostrongyle: *Marshallagia marshalli*, *Teladorsagia circumcincta,* and *Ostertagia occidentalis*) in ungulates, since host resistance influences parasite fecundity [50]. Faecal egg counts were done following a modified McMaster technique [51] and were expressed as number of eggs per gram of fresh faeces (EPG). We calculated the average EPG for each animal during the season. For more details see Brambilla et al. [46].

Data analysis

Hardy-Weinberg equilibrium (HWE) was calculated for all the microsatellites using the Microsoft Office Excel ® plugin GeneAlEx 6.5 [52]. Loci not in HWE after Bonferroni sequential correction [53] were excluded from further analysis. Standardized multilocus heterozygosity (MLH) was then calculated for each animal as the ratio of the heterozygosity of the individual to the mean heterozygosity of those loci at which the individual was typed. The standardization avoids

194 confounding because of possible systematic differences in loci used between individuals [54]. To
195 minimize the risk of type I error from multiple tests, all probabilities were corrected with the
196 sequential Bonferroni method [53]. Since we used the genotypes of animals born between 1985 and
197 2009, to exclude directional cohort effects, we fitted a linear regression to test changes in MLH over
198 time.
199
200 We estimated g_2, a measure of the covariance in heterozygosity, using the freeware software RMES
201 (Robust Multilocus Estimates of Selfing) [55]. RMES, besides providing an estimate of g_2 also tests
202 whether g_2 differs significantly from zero. Values of $g_2 = 0$ mean that there is no variance in
203 inbreeding in the population and thus HFCs are not expected to arise [13]. The analysis was
204 performed setting in RMES the number of populations equal to one, n= 37 useful microsatellites
205 and running it for 1000 iterations.
206
207 To test the relationship between MLH and fitness related traits, we fitted linear mixed effect models
208 using the 'lmer' function in package lme4 [56] in R 3.0.0 [57]. All the variables were standardized
209 before analysis to obtain comparable measures of the effect size of MLH on the analyzed trait. We
210 included age and age^2 of individuals as well as the interaction between age and MLH as fixed
211 covariates in models testing the effect of MLH on body mass and horn growth as these traits are
212 known from previous analyses to be age dependent following a quadratic curve [48,58]. The effect
213 of MLH on FEC was modeled adding age and the interaction between age and MLH as fixed
214 covariates. As we had repeated measures for individuals and measures taken in different years,
215 individual identity and year were added as random effects in all the models. A set of models with all
216 the possible combinations of the variables of the full model were fitted for each fitness trait by
217 testing the relative importance of the variables using the dredge function of the R package MuMIn
218 [59]. Coefficients were estimated model averaging among all fitted models using the model.avg

function in package MuMIn. Model selection was finally done comparing corrected values of Akaike's Information Criterion, AICc [60]. A value of ΔAICc= 4 was chosen as a threshold for the selection of the best models [61]. Pseudo-R^2 values were calculated for the four best models with the r.squaredGLMM function of the package MuMIn. Marginal R^2 (R^2m) represents the variance explained by fixed factors, conditional R^2 (R^2c) is interpreted as variance explained by both fixed and random factors.

Since we hypothesized that body mass, horn growth and FEC were not independent, after testing the correlation between MLH and fitness related traits we tested alternative hypothesized causal models of the relationship among variables using confirmatory path analysis with the d-sep method [22,24,62,63]. Following our hypothesis we tested six different causal models represented as directed acyclic graphs in Figure 1: model a) with body mass, horn growth and FEC as directly being affected by MLH; model b) with MLH having both a direct effect and an indirect effect mediated by body mass on horn growth; model c) with MLH having no direct effect on horn growth but only the indirect effect mediated by body mass; model d) with an indirect effect of MLH on horn growth mediated by body mass but no direct effect on FEC; model e) with an indirect effect of MLH on horn growth mediated both by body mass and FEC; model f) with an indirect effect of MLH on horn growth mediated by body mass only and no direct effect of MLH on FEC, which instead affects horn growth directly. Independence claims declared to describe the hypothesized causal models were tested using linear mixed models including individual identity and year as random factors as described by Shipley [62]. Following Shipley [22,24], the data were considered as consistent with the hypothesized causal model if the *p*-value was > 0.05. Competing causal models were also compared using AICc, following the procedure for path analysis with hierarchically structured data suggested by Shipley [62]. The number of parameters (*d.f.*) for each structural equation forming part of the hypothesized causal model, needed to calculate AICc, was

244 extracted from the 'lmer' models using the function 'logLik' in R.

245

246 Results

247 Dropout and false allele rates, provided as supplementary material (Table S1), were similar to what
248 was found in other studies on the same species using the same microsatellite loci [30]. False allele
249 rates were 0 for all the markers analyzed, while dropout ranged between 0 and 0.172 (mean ± S.D.
250 = 0.012 ± 0.028).
251 After Bonferroni correction, four markers were not in HWE (INRA175, OarAE54, BM2113 and
252 SR-CRSP07). Three of these four markers (INRA175, OarAE54, BM2113), however, were
253 consistently in HWE in 43 populations previously analyzed [30]; since an analysis of multiple
254 populations should be more precise in finding markers not in HWE than a single population
255 analysis like our study, we decided to keep these three markers in our analysis and to exclude only
256 SR-CRSP07. We also did not include 13 markers in the analysis known to be linked to loci under
257 selection (i.e. markers within or linked to MHC or to other immune genes, markers linked to known
258 quantitative traits loci, or markers that deviated from the neutral expectation [30]. We thus
259 calculated MLH from 37 markers all supposed to be neutral (Table S1 supplementary materials).
260 There was no correlation between MLH and the cohort of individuals. This result, therefore, does
261 not support the hypothesis of cohort effects or changes in MLH over time (linear regression: $\beta \pm$
262 S.E. = 0.002 ± 0.003 $p = 0.420$, n=149, Figure S3 of supplementary materials).
263 The estimate of g_2 was not significantly different from 0 ($g_2 \pm$ S.D. = -0.0022 ± 0.0039, $p = 0.702$).
264 The relatively large standard deviation of the estimate suggests that g_2 was estimated with low
265 precision in our study.

266

267 *Heterozygosity-Fitness Correlations:*

268 Body mass

The best models included both MLH, age and the interaction between MLH and age (Table 1) with MLH having a weak, positive relationship with body mass (model averaged coefficient β ± S.E. = 0.043 ± 0.058) and the interaction between age and MLH having a weak, negative correlation with body mass (model averaged coefficient β ± S.E. = -0.060 ± 0.033). R^2m and R^2c of the best models are presented in Table 1.

Horn growth

The best models included MLH, age, and the interaction between MLH and age with a weak, positive relationship between MLH and annual horn growth (model averaged coefficient β ± S.E. = 0.080 ± 0.040) and a weak, negative relationship between the interaction between MLH and age and horn growth (model averaged coefficient β ± S.E. = -0.067 ± 0.033; Table 2). R^2m and R^2c of the best models are presented in Table 2.

Faecal Egg Counts

The best models included both MLH and age (Table 3) with MLH having a weak, negative relationship with FEC (MLH: model averaged coefficient β ± S.E. = -0.087 ± 0.053). R^2m and R^2c of the best models are presented in Table 3.

Confirmatory Path analysis:

The only causal model consistent with the data among those we tested with confirmatory path analysis (i.e. the only with a *p*-value > 0.05) was model e), which implies an indirect effect of MLH on horn growth mediated by both body mass and FEC (Figure 2, Table 4). The ΔAICc values confirmed that this was indeed the best fitting causal model (Table 4). Details on the independence claims describing each of the six models as well as on model testing are provided in the supplementary material (Tables S2, S2.1-S2.6).

Discussion

Our results revealed heterozygosity-fitness correlations in all analyzed fitness-related traits in male Alpine ibex. We found a positive relationship between MLH and body mass, with more heterozygous individuals being heavier. The same relationship was found for horn growth, with more heterozygous individuals having longer annual horn growth. In the case of faecal egg counts, the relationship instead was negative, as expected, with less heterozygous individuals having the highest FEC.

Direct and indirect causal relationships between individual genetic variability and life history traits are not easy to disentangle, and the effect of MLH on one traits may actually be mediated by some other trait. Indeed, the traits used to assess individual quality, are often not independent: heavier males are presumably of higher quality, and they may also be able to afford growing bigger horns [64]. Using confirmatory path analysis we showed that the effect of MLH on horn growth was not direct, but was instead mediated by body mass and FEC: only high quality males (i.e. with high levels of heterozygosity) become big (large body mass) and resistant to parasites (low FEC) and consequently can afford to also grow long horns. This result supports the hypothesis that horn growth in male Alpine ibex is an honest advertisement of individual quality [65] as suggested by von Hardenberg et al. [38]. Our final causal model contradicts the hypothesis of a direct relationship between faecal egg counts and body mass previously found in the same population by Decristophoris et al. [66]. However, that study [66] did not include data on MLH, while the causal model presented here, including this third variable, shows that the relationship originally found between FEC and body mass is actually due to the direct causal effect of MLH on both traits. As a side result, this suggests that faecal egg counts may be a proxy of parasite resilience rather than resistance [67]: the effect of MLH both on body mass and FEC may actually be mediated by another unmeasured variable such as, for example, by the ability to obtain forage of better quality (in terms of protein content) [68]. Indeed, increased individual resilience towards gastrointestinal

parasites was shown to be related to the quality of the forage in domestic goats experimentally infected with abomasal parasites [69], while high protein diet supplementation improved both resilience and resistance to gastrointestinal parasites in domestic sheep lambs [67].

Our results are in accordance with von Hardenberg et al. [38], who, in the same population, found weak age-specific effects of MLH on horn growth. The broader effects that we found here are probably due to the fact that the present study relies on a considerably larger data set of fitness-related traits compared to the von Hardenberg et al. [38] study, which only included data from 2000 to 2004. Moreover, the confirmatory path analysis approach that we used here allowed us to demonstrate that the effect of heterozygosity on horn growth, found also previously, was not direct but mediated by body mass. We also tested changes in MLH over time to possibly explain the different findings between our study and the previous one [38], but the increase in MLH in the Gran Paradiso population was small and non-significant over the last 25 years suggesting that there has not been a substantial recent increase in inbreeding. This finding is in line with the relatively large population size of this population in recent years (n= 2651 individuals counted during the yearly total count in September 2013, Data provided by GPNP Scientific Research Service).

The magnitude of the effects of MLH on fitness-related traits that we detected was weak, in accordance with the findings of many other studies [12,13,20,70]. The effect of MLH on fitness-related traits appeared to be age dependent: the effect of MLH on body mass and annual horn growth decreased with age. This can be explained by the fact that growth differences are maximal early in life [71]. Following David et al. [71], there may be higher variance in fitness related traits in young compared to old individuals since unfit genotypes are selectively eliminated in older cohorts and thus it may be easier to detect the effect of MLH in young compared to old individuals. On the other side, fecal egg counts decreased with increasing MLH while they increased with age, but, contrary to the other considered fitness traits, the two effects did not appear to interact.

Even if we did not use direct fitness measures, as suggested by Chapman et al. [12], the traits we

chose to test for HFCs seem appropriate. Body mass and horn growth have an effect on the dominance status of male Alpine ibex [64] and, thus, probably contribute to reproductive success [72]. Secondary sexual traits are also known to be honest signals of individual quality [58,73]. Gastrointestinal parasites, as well, have a crucial role in the life history and survival of wild herbivores and create repercussions on population dynamics [74-78]. Other authors found evidence of HFCs in ungulates using morphological and physiological traits; these studies, however, usually analysed the effect of MLH on single traits [see for example: 10,39,54,79]. In this study, instead, we show a broad effect of multilocus heterozygosity on several traits at once in accordance with the general effect hypothesis for HFCs [12].

Almost two hundred years have passed since the main bottleneck of this population [31]. Thus, about 25 Alpine ibex generations have passed since this bottleneck. During the Second World War, the Gran Paradiso population suffered an additional numerical reduction with no more than 600 animals left (Gran Paradiso National Park Archives, unpublished data). Since the first bottleneck, inbreeding due to small population size has probably accumulated and heterozygosity has been lost. While we do not have direct evidence of this loss of heterozygosity in the Gran Paradiso population, this effect has been recently demonstrated in reintroduced Alpine ibex populations in Switzerland [37]. The magnitude of the increase in inbreeding and loss in heterozygosity each generation varies proportionally to the reciprocal of the effective population size [80]. The two bottlenecks might have contributed most to the inbreeding coefficient, and further inbreeding may have accumulated also in the following generations due to the small numbers of effective individuals in the population. Heterozygosity appears to reflect inbreeding in the Gran Paradiso population: Szulkin et al.'s [13] test for local effects did not reveal any evidence for local effects since the model containing specific effects for each locus did not differ from the model containing MLH (χ^2 = 46.087, df = 36, p = 0.121), suggesting that the HFCs we found are more likely due to general effects. Hence, our results are most parsimoniously interpreted as inbreeding depression, with a

369 fitness advantage for less inbred individuals.

370 Contrary to our expectations, however, we found no evidence of identity disequilibrium (ID) with
371 g_2 not significantly different from zero. As ID is considered a definite consequence of inbreeding
372 and the proximal cause of HFCs, in absence of ID (if $g_2 = 0$), HFCs are not expected to arise [13].
373 Indeed, following the formula proposed by Miller et al. [81], when $g_2 = 0$, the power to detect HFCs
374 becomes 0 as well. However, other studies found HFCs despite having no evidence for ID [10,82].
375 The number of genotyped markers used in our and other studies, may in fact be too small to reliably
376 detect ID: weak levels of inbreeding depression sometimes can be detected more easily through an
377 analysis at phenotype level than by using genotype indexes based on a few microsatellites [13]. Our
378 results mirror those of a recent simulation study [83], which found that only a small proportion of
379 populations with significant HFCs exhibited significant ID when either a small number of markers
380 was used or when the variance in inbreeding was low. Further analyses using a larger number of
381 markers (for example using SNPs) might help to better understand HFCs in this population [81].
382 HFCs found in this study provide evidence for inbreeding depression in the Gran Paradiso Alpine
383 ibex population. The genetic bottlenecks suffered by this population [36] lead to genetic drift, which
384 in turn could lead to the fixation of deleterious alleles [1]. However, the detection of inbreeding
385 depression indicates that there is still genetic variability in fitness-related traits in this population
386 and, thus, that deleterious alleles are not all fixed in the Alpine ibex in Gran Paradiso National Park.
387 The persistence of inbreeding depression many years after the bottleneck is also extremely
388 interesting from a conservation point of view. The Gran Paradiso population is the only one which
389 survived the 19th century while all the other populations extant today are derived from
390 reintroduction programs [30]; this population was, thus, expected to be the least affected by
391 inbreeding, because it experienced fewer bottlenecks during its population history. Since the
392 autochthonous Gran Paradiso population presents signs of inbreeding depression, it would be very
393 important to quantify inbreeding depression in the more recently founded populations which are

394 already known to be more inbred than the Gran Paradiso population [37]. Furthermore, we have
395 shown the usefulness of confirmatory path analysis [22] in the field of molecular ecology to test
396 causal models of the relationships between individual genetic variability and fitness-related traits.
397 Specifically, we were able to show that the previously found positive relationship between MLH
398 and horn growth in Alpine ibex [38] is not direct but is rather mediated through body mass and
399 parasite resistance and resilience. Disentangling correlations from causation among heterozygosity
400 and fitness-related traits helped us to highlight the mechanisms behind HFCs and to explain the
401 different effect size of MLH on different traits [84]. We thus believe that confirmatory path analysis
402 and more generally causal inference methods [23], should become part of the statistical toolbox of
403 molecular ecologists, especially of those working on long term studies on wild populations in which
404 the possibilities of experimental manipulation are usually very limited.

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406

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416

417 Data accessibility

418 The data used in this paper is published on Dryad, doi:10.5061/dryad.8kb87

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637 Tables

638 Table 1: Model selection of mixed effects models for testing the effect of MLH and age on body
639 mass, n= 391. ID and year were fitted as random effects. Weight is the Akaike weight on which
640 model averaged coefficients are based. R^2m and R^2c represent marginal and conditional pseudo-R^2
641 values respectively. Only the best four models are shown.

components of the model	*df*	log likelihood	AICc	Δ AICc	weight	R^2m	R^2c
Age + Age^2 + MLH	7	-227.18	468.67	0	0.87	0.61	0.92
Age + Age^2 + MLH + Age*MLH	8	-228.06	472.52	3.85	0.13	0.62	0.91
Age + Age^2	6	-238.27	488.75	20.08	0	0.62	0.91
Age + MLH	6	-349.88	711.99	243.32	0	0.49	0.77

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Table 2: Model selection of mixed effects models for testing the effect of MLH and age on horn growth, n= 873. ID and year were fitted as random effects. Weight is the Akaike weight on which model averaged coefficients are based. R^2m and R^2c represent marginal and conditional pseudo-R^2 values respectively. Only the best four models are shown.

components of the model	*df*	log likelihood	AICc	Δ AICc	weight	R^2m	R^2c
Age + Age^2 + MLH	7	-989.28	1992.7	0	0.81	0.34	0.43
Age + Age^2 + MLH + Age*MLH	8	-989.71	1995.59	2.88	0.19	0.34	0.43
Age + MLH	6	-1006.6	2025.29	32.59	0	0.30	0.40
Age + MLH + Age*MLH	7	-1008.93	2032	39.29	0	0.30	0.40

651 Table 3: Model selectionof mixed effects models for testing the effect of MLH and age on FEC,
652 n=510. ID and year were fitted as random effects. Weight is the Akaike model weight on which
653 model averaged coefficients are based. R^2m and R^2c represent marginal and conditional pseudo-R^2
654 values respectively. Only the best four models are shown.

components of the model	*df*	log likelihood	AICc	Δ AICc	weight	R^2m	R^2c
Age + MLH	6	-591.49	1195.16	0	0.95	0.10	0.47
Age + MLH + Age*MLH	7	-593.43	1201.1	5.94	0.05	0.10	0.47
MLH	5	-610.88	1231.89	36.73	0	0.02	0.04
Age	5	-643.5	1297.12	101.95	0	0.10	0.48

655

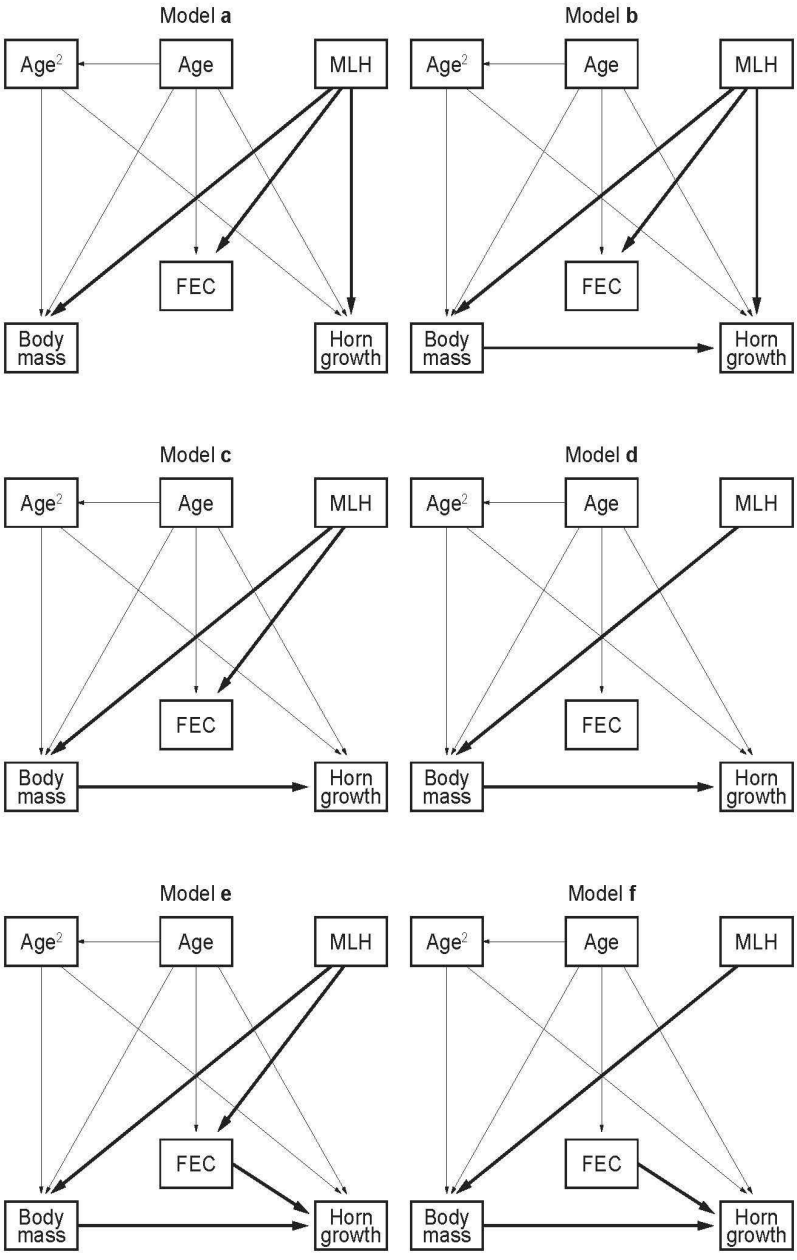
Table 4: Summary of the confirmatory path analysis results for the six hypothetical causal models. Models are presented in order of AICc. *C* represents Fisher's C statistics [22]. The directed acyclic graphs of all the six models are represented in Figure 1. Model e, the only accepted model after model selection, is represented in Figure 2 with standardized path coefficients. Basis sets with all implied conditional independence claims for all models are described in supplementary material S2 (S2.1-S2.6).

model	***df***	***C***	***p-value***	**AICc**	**Δ AICc**
e	**26**	**17.58**	**0.063**	**69.58**	**0.00**
c	25	26.31	0.010	76.31	6.73
b	26	25.54	0.004	77.54	7.96
f	24	45.59	<0.001	93.59	24.01
a	25	49.27	<0.001	99.26	29.68
d	24	54.29	<0.001	102.29	32.71

665 Figures

666

667 Figure 1: Representation of the six models testing causal relationships between variables in HFCs.
668 MLH represents individual standardized multilocus heterozygosity and FEC represents faecal egg
669 count, a measure of individual parasite resistance and resilience. The thicker arrows were used to
670 highlight the causal links that change among the six models.



672 Figure 2: Representation of the causal pathways of the best fitting model (model e) with

673 standardized path coefficients represented within circles.